Novel Tocotrienols of Rice Bran Suppress Cholesterogenesis in Hereditary Hypercholesterolemic Swine[1]

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ABSTRACT A tocotrienol-rich fraction (TRF_{25}) and novel tocotrienols (d-P_{21}-T3 and d-P_{25}-T3) of rice bran significantly lowered serum and low density lipoprotein cholesterol levels in chickens. The present study evaluated the effects of novel tocotrienols on lipid metabolism in swine expressing hereditary hypercholesterolemia. Fifteen 4-mo-old genetically hypercholesterolemic swine were divided into five groups ($n = 3$). Four groups were fed a corn-soybean control diet, supplemented with 50 μg of either TRF_{25}, γ-tocotrienol, d-P_{21}-T3 or d-P_{25}-T3 per g for 6 wk. Group 5 was fed the control diet for 6 wk and served as a control. After 6 wk, serum total cholesterol was reduced 32–38%, low density lipoprotein cholesterol was reduced 35–43%, apolipoprotein B was reduced 20–28%, platelet factor 4 was reduced 12–24%, thromboxane B_2 was reduced 11–18%, glucose was reduced 22–25% ($P < 0.01$), triglycerides were reduced 15–19% and glucagon was reduced 11–17% ($P < 0.05$) in the treatment groups relative to the control. Insulin was 100% greater ($P < 0.01$) in the treatment groups than in the control group. Preliminary data ($n = 1$) indicated that hepatic activity of the 3-hydroxy-3-methylglutaryl–coenzyme A reductase was lower in the treatment groups, and cholesterol 7α-hydroxylase activity was unaffected. Cholesterol and fatty acid levels in various tissues were lower in the treatment groups than in control. After being fed the tocotrienol-supplemented diets, two swine in each group were transferred to the control diet for 10 wk. The lower concentrations of serum lipids in these four treatment groups persisted for 10 wk. This persistent effect may have resulted from the high tocotrienol levels in blood of the treatment groups, suggesting that the conversion of tocotrienols to tocopherols may not be as rapid as was reported in chickens and humans. J. Nutr. 131: 223–230, 2001.

KEY WORDS: • *hereditary hypercholesterolemic (HH) swine* • *rice bran* • *novel tocotrienols* • *lipid metabolism* • *cholesterol metabolism*

The impact of pure tocotrienols isolated from barley, rice bran and palm oil on cholesterol biosynthesis, specifically through the lowering of LDL cholesterol (LDL-chol)[3] and inhibition of 3-hydroxy 3-methylglutaryl–coenzyme A (HMG-CoA) reductase, has been confirmed by a number of investigators (Hood 1995, Hood and Sidhu 1992, Khor et al.1995, Parker et al. 1993, Qureshi et al. 1997, Qureshi and Qureshi 1993, Watkins et al. 1993). Furthermore, the hypocholesterolemic effects of rice bran have been demonstrated in experimental animals (Kahlon et al. 1991, Seetharamaiah and Chandrashekhara 1989, Sharma and Rukmini 1987). This lowering of serum total cholesterol (total-chol) levels by rice bran was initially attributed to constituents of the unsaponifiable fractions of the oil, such as cycloartenol (triterpene alcohols) or ferulic acid esters (γ-oryzanols), which are present in large amounts (Kahlon et al. 1991, Seetharamaiah and Chandrashekhara 1989, Sharma and Rukmini 1987). However, we have demonstrated that tocotrienols, minor constituents present in various cultivars of rice brans, exert powerful hypocholesterolemic effects in humans and chickens (Qureshi et al. 1997 and 2000). The lipid variables and lipoprotein profiles of swine are more similar to those of humans than to those of rats (Hasler-Rapacz et al. 1995, Huang and Kummerow 1976, Leveille et al. 1975, Shrago et al. 1971).

A strain of swine that expresses spontaneous hyperlipidemia and hypercholesterolemia (hereditary/genetically) is derived from a unique gene pool population that has resulted from a worldwide survey of 37 breeds and subsequent experimental breeding of uncommon lipid phenotypes and lipoprotein genotypes (Rapacz 1978). The serum total-chol concentrations in these swine are 7.76–12.93 mmol/L compared with

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[3] Abbreviations used: apo, apolipoprotein; HH, hereditary hypercholesterolemic; HMG-CoA, 3-hydroxy 3-methylglutaryl–coenzyme A; LDL-chol, low density lipoprotein cholesterol; PF 4, platelet factor 4; d-P_{21}-T3, desmethyl tocotrienol from stabilized and processed rice bran; d-P_{25}-T3, didesmethyl tocotrienol from stabilized and heated rice bran; total-chol, total cholesterol; RIA, radioimmunoassay; d-TRF_{25}, tocotrienol-rich fraction from stabilized and heated rice bran; TXB_2, thromboxane B_2.

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3.10–4.14 mmol/L in normal adult swine. The phenotype in these swine shows twofold to fourfold elevated concentrations of serum or plasma lipids [total-chol, LDL-chol, triglycerides (TG)] and apolipoprotein (apo)B, apoC3 and apoE and reduced levels of HDL cholesterol (HDL-chol), apoA1 and apoA4 (Hasler-Rapacz et al. 1995). Plasma from these swine is characterized by cholesterol ester–enriched buoyant LDL (Lee et al. 1990) as well as dense LDL particles (Rapacz and Hasler-Rapacz 1984). These hereditary hypercholesterolemic (HH) swine develop complicated atherosclerotic plaques that closely resemble advanced atherosclerotic lesions found in humans (Hasler-Rapacz et al. 1995, Prescott et al. 1995). Therefore, these swine are a good model for studying the effects of tocotrienols from rice brans on LDL-chol and other lipid parameters. High concentrations of serum LDL-chol or apoB and low concentrations of HDL-chol or apoA1 are found to be better predictors for the severity of coronary artery stenosis than are total-chol and TG concentrations. Therefore, the ratios of HDL-chol to LDL-chol and apoA1 to apoB are used as indicators for assessment of the risk for coronary heart disease (Naito 1986, Vega and Grundy 1990).

The present study was carried out to evaluate the effects of tocotrienol-rich fraction (TRF_{25}) and its individual components, γ-tocotrienol, desmethyl (*d*-P_{21}-T3) and didesmethyl (*d*-P_{25}-T3) tocotrienols, in HH swine. The TRF_{25} is mainly a mixture of α-, γ- and δ-tocotrienols plus two novel tocotrienols, *d*-P_{21}-T3 and *d*-P_{25}-T3, isolated and purified from stabilized and heated rice brans by flash chromatography (Qureshi et al. 2000).

It has been established that the relative contributions of various tissues to overall fatty acids and cholesterol synthesis vary among animal species (Dietschy et al. 1993). All of the fatty acid synthesis takes place in the liver of chickens and humans, whereas in rats and swine, adipose tissue is a major contributor (Huang and Kummerow 1976, Leveille et al. 1975, Shrago et al. 1971). In swine, cholesterol biosynthesis supported by acetate or glucose occurs mainly in the liver and to some extent in adipose tissue (Huang and Kummerow 1976); however, the lipid parameters and profiles in swine are similar to those of humans (Hasler-Rapacz et al. 1995, Huang and Kummerow 1976, Leveille et al. 1975, Shrago et al. 1971). The present study describes the effects of TRF_{25}, γ-T3, *d*-P_{21}-T3 and *d*-P_{25}-T3 fed to HH swine on the levels of serum total-chol, LDL-chol, HDL-chol, apoA1, apoB, thromboxane B_2 (TXB_2), platelet factor 4 (PF4), glucose, insulin and glucagon, as well as on the hepatic enzymatic activities of HMG-CoA reductase and cholesterol 7α-hydroxylase. The concentrations of cholesterol and the fatty acid compositions were also determined in liver, intestines, lungs, heart, loin muscles, adipose tissues and ham muscles in the experimental and control HH swine.

MATERIALS AND METHODS

Sources of chemicals and diagnostic kits. Sources of chemicals, substrates, labeled substrates, enzymes and diagnostic kits have been identified previously (Qureshi et al. 1997 and 2000). Chemicals and solvents were of analytical grade. Sigma Diagnostic Kits (Sigma Chemical Co., St. Louis, MO) were used to estimate serum total-chol (kit 352; 500 nm), LDL-chol (kit 352; 500 nm), HDL-chol (kit 352; 500 nm), TG (kit 336; 500 nm), apoAI (kit 356-A; 340 nm), apoB (kit 357; 340 nm) and glucose (kit 315; 505 nm). PF4 was determined by using Elisa Kit for PF4 (kit 601; American Bioproduct, Parsippany, NJ), and a radioimmunoassay (RIA) kit was used to determine TXB_2 levels (Chemicon International, El Segundo, CA). Serum insulin concentrations were estimated by using a Double Antibody RIA Kit (Ventrex Laboratories, Portland, OR), and glucagon was estimated by using an RSL Glucagon Kit (ICN Biomedicals, Costa Mesa, CA).

Purification of γ-oryzanol–free TRF_{25} by flash chromatography. The stabilized and heated rice brans of L-487 rice variety were supplied by M. Wells (Riviana Foods, Abbeville, LA). Large quantities of tocotrienol-rich fraction (TRF_{25}) and its individual tocols (tocopherols and tocotrienols) were prepared by extracting 10 batches (10 kg of each) of stabilized and processed rice bran (L-487 variety; tocols 476 μg/g) twice with methanol (20 L) in a stainless steel pan (50 L). The mixture was stirred six times during the day with a stainless steel spoon for 3 d. The methanol (selective extracts polyphenols, tocols, sterols, γ-oryzanols, fatty acids esters and some phospholipids)-soluble fraction was separated by filtering over a pad of four layers of cheese cloth. The combined methanol fractions from all 10 batches were dried under vacuum (50°C). The semiviscous oily residue (4600 g) of the methanol-soluble fraction was extracted with hexane (5 L), filtered and left at 0°C overnight to remove some of the sterols and triacylglycerides as precipitates. The hexane-soluble fraction was filtered and evaporated under vacuum at 40°C, yielding 2412 g of a mixture of TRF_{25}, γ-oryzanols, some low-molecular-weight polyphenols, some free fatty acids, fatty acid esters and triacylglycerides. HPLC of viscous oil for tocols revealed the presence of α-tocopherol, α-tocotrienol, β-tocotrienol, γ-tocotrienol, δ-tocopherol, δ-tocotrienol and two novel tocotrienols (*d*-P_{21}-T3 and *d*-P_{25}-T3). The molecular structures of *d*-P_{21}-T3 and *d*-P_{25}-T3 have been established as 3,4-dihydro-2-methyl-2-(4,8,12-trimethyltrideca-3′(*E*),7′(*E*),11′-trienyl)-2*H*-1-benzopyran-6-ol and 3,4-dihydro-2-(4,8,12-trimethyltrideca-3′(*E*),7′,(*E*),11′-trienyl)-2*H*-1-benzopyran-6-ol, respectively (Qureshi et al. 1999 and 2000). The purification of large quantities of TRF_{25} free from γ-oryzanols and most of α-tocopherol was carried out by flash chromatography, using silica gel as reported recently (Qureshi et al. 2000).

The tocols of TRF_{25} mixture were identified according to the retention time and absorption profiles against standards of tocols described earlier (Qureshi et al. 2000). The composition of various tocols in TRF_{25} was 4.5% α-tocopherol, 15.7% α-tocotrienol, 2.3% β-tocotrienol, 34.6% γ-tocotrienol, 4.8% δ-tocopherol, 7.9% δ-tocotrienol, 13.7% *d*-P_{21}-T3, 14.4% *d*-P_{25}-T3 and 2.1% unidentified tocotrienols.

Purification of γ-tocotrienol, d-P_{21}-T3 and d-P_{25}-T3 by flash chromatography. The purification of γ-tocotrienol, *d*-P_{21}-T3 and *d*-P_{25}-T3 was carried out with the remaining viscous oil (1912 g) by using the same procedure as described previously (Qureshi et al. 2000), except various tocols were eluted with the sequential application of 1 L diethyl ether (DE) (5, 10, 12, 14, 16, 18, 20, 22, 25 and 30% v/v) in hexane. The composition of the combined fractions obtained from 19 pools (1912 g starting material) with each elution was 5% DE; fatty acids esters and sterols (1266 g); 10% DE, α-tocopherol (3.3 g); 12% DE, a mixture of α-tocopherol and α-tocotrienol (2.7 g); 14% DE, α-tocotrienol (3.7 g); 16% DE, β-tocotrienol (0.4 g); 18% DE, γ-tocotrienol (11.6 g); 20% DE, a mixture of δ-tocopherol and δ-tocotrienol contaminated with *d*-P_{21}-T3 (3.9 g); 22% DE, *d*-P_{21}-T3 (4.7 g); 25% DE, *d*-P_{25}-T3 (2.1 g); and 30% DE, *d*-P_{25}-T3 (4.1 g). Washing with 100% DE or methanol gave γ-oryzanols (29.4 g). Each of these fractions contained > 95% of its major component of the respective tocol.

Animals. The protocol was reviewed and approved by the University of Wisconsin-Madison College of Agriculture and Life Sciences Animal Care Committee.

The HH swine used in this study were from the Immunogenetic Project Herd, University of Wisconsin, Madison. The swine were derived from the gene pool population and were selected based on the cholesterol phenotypes (total-chol, HDL-chol) determined at 5 mo of age and immunogenetically defined lipoprotein polymorphisms (Hasler-Rapacz et al. 1995). They represented both sexes and were fed a standard 20% protein, corn-soybean meal swine (control, tocotrienols 0.0%) diet as reported previously (Qureshi et al. 1991a), formulated at the University of Wisconsin Experiment Station. The control diet contains 717.7 g corn (9.3%), 210 g soybean meal (44%), 30 g lard, 11.3 g dicalcium phosphate, 8 g calcium carbonate, 3 g NaCl and 10 g mineral-vitamin premix (Qureshi et al. 1991a). The National Research Council guide for the care and use of laboratory

animals was followed. Fifteen 20-wk-old HH and hereditary hyperlipidemic swine were divided into five groups ($n = 3$, two males and one female), and each group of three was placed into an individual 2.5 × 2.5-m pen. Four of these five groups represented the treatment groups, and the fifth served as the control group. The treatment diets were prepared by supplementing the standard 20% protein, corn-soybean meal (control) diet as described here (Qureshi et al. 1991a) with 50 μg of either TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3 per d and fed for 42 d. A dose of 50 μg/g for each treatment was used in the present study, which was very effective in inhibiting the activity of HMG-CoA reductase in chickens (Qureshi et al. 2000). Swine consumed feed and water ad libitum. Feed intake by pen and individual body weights was recorded weekly. Six normolipemic swine (20-wk-old; four males and two females), derived from the Nutrition Project, University of Wisconsin-Madison, were included to compare the weight gain and other variables at 21, 42 and 70 d of feeding with that of the HH/hereditary hyperlipidemic swine.

Blood samples, drawn by a syringe from the jugular vein, were taken on d 0, 21 and 42 of feeding after overnight food deprivation to facilitate chylomicron and very low density lipoprotein clearance. The blood was collected (5 mL each time), processed for serum or plasma and kept at −20°C for blood chemistry analyses. After being fed the diets for 42 d and having blood drawn after overnight food deprivation, the swine were further starved for 36 h. Then the swine were refed for 48 h, and one swine (male, randomly selected) from each group and two males and one female from the normolipemic group were killed according to procedures approved by the Research Animal Resources Center (RARC), University of Wisconsin-Madison. Samples of liver, intestine, lung, heart, loin muscle, adipose tissue and ham muscles were collected at the Muscle Biology Laboratory, University of Wisconsin, and stored at −20°C for subsequent analyses.

In addition, livers (15–20 g) were washed with saline solution, placed on ice and processed to prepare cytosolic and microsomal fractions to carry out cholesterol biosynthesis assays (Qureshi et al. 1991a and 1982). The food deprivation and refeeding segments of this treatment were performed to induce hepatic HMG-CoA reductase (EC 1.1.1.34) and cholesterol 7α-hydroxylase (EC 1.14.13.17) activities as described here (Leveille et al. 1975). The remaining two swine (one male and one female of the hypercholesterolemic group and two males and one female of the normolipemic group) were transferred to a corn-soybean control diet for 10 wk, and serum and plasma samples prepared from blood were collected after overnight food deprivation for lipid analyses. The microsomal fractions of liver, serum and plasma samples were stored at −20°C for subsequent analyses. Feed consumption and feed efficiencies were calculated from body and feed weights taken at the start and the end of the experiment.

Serum lipid analyses and apolipoprotein assays. Serum total-chol and TG were assayed using kits 352 and 336, respectively. LDL-chol and very low density lipoprotein cholesterol were precipitated using 400-μL aliquots of serum with 50 μL of a mixture of 9.7 mmol phosphotungstic acid and 0.4 mol $MgCl_2$ per L with gentle shaking for 10 min at room temperature, followed by centrifugation at 12,000 × *g* for 10 min. The supernatant, containing HDL-chol, was analyzed with Sigma reagent (kit 352). LDL-chol was calculated according to the formula LDL-chol = (total chol) − (HDL-chol) − (TG/5). Serum apoA1 and apoB were determined by RIA using antibodies to humans apoA1 and apoB (Sigma kits). Similarly, plasma PF4 and TXB_2 concentrations were determined with RIA kits. Triplicates of each sample were run analyzed in each assay, and the average was used for the final calculation.

Estimation of tissue cholesterol levels and fatty acid composition. The estimation of cholesterol in various tissues was carried out by homogenizing each tissue (0.2 g) in sodium citrate buffer (0.1 mol/L; 1 mL) with a microprobe from Omni GLH International Homogenizer (Warrenton, VA) for 30–60 s; 10 μL of the emulsion was used to estimate the cholesterol as described here with Sigma diagnostic reagent kit 352.

The fatty acid compositions were determined as described by Hirai et al. (1984). Each tissue (1 g) was homogenized for 30 s with a Polytron homogenizer in 2 mL saline solution. After homogenization, the sample was extracted with 8 mL hexane by shaking for 20 min and then centrifuging for 10 min at 12,000 × *g*. The hexane layer was dried at 40°C under vacuum, and the residue was treated with 0.5 mL diazomethane to yield the fatty acid esters. The levels of fatty acids were measured as their esters by using a Hewlett Packard model 5710A gas chromatograph equipped with automatic sampler 7672A (Hewlett Packard, Boise, ID). The identification of each fatty acid ester was established by comparison against a standard mixture of fatty acid esters (Sigma Chemical Co.). Triplicates of each sample were included in each assay, and the average was used for the final calculation.

Assays for cholesterol biosynthesis. Hepatic enzyme activities were assayed in the microsomal fraction of liver homogenates. Fresh livers were homogenized and the microsomes were obtained through centrifugation as described previously (Qureshi et al. 1982 and 1991a) and stored at −20°C until used for assays. The liver homogenates were prepared in 0.1 mol potassium phosphate/L buffer, pH 7.4, containing 4 mmol $MgCl_2$, 1 mmol EDTA and 2 mmol dithiothreitol per L (Qureshi et al. 1991a and 1982). Microsomal protein was measured by the Biuret method with bovine serum albumin used as a standard. HMG-CoA reductase activity was assayed from the microsomal fractions by the production of radioactive mevalonic acid from *dl*-3-hydroxymethyl-[3-^{14}C]glutaryl-CoA. Likewise, cholesterol 7α-hydroxylase activity was assayed from microsomal fractions by the production of [^{14}C]7α-hydroxycholesterol from [^{14}C]cholesterol (Qureshi et al. 1991a and 1982). Aquasol scintillation solution, *dl*-3-hydroxy-3-methyl-[3-^{14}C]glutaryl-CoA and [^{14}C]cholesterol were purchased from New England Nuclear (Boston, MA). An average of triplicate analyses of each sample was used for these two enzymatic assays. Enzyme data are presented as specific activities.

Expression of data and statistical methods. StatView software (1992; Abacus Concepts, Berkeley, CA) was used for the analysis of treatment-mediated effects. Treatment-mediated differences in serum lipid variables were identified with ANOVA, and when the *F* test indicated a significant effect, the differences between the means were analyzed by a Fisher's Protected Least Significant Difference (LSD) test (1992; Abacus Concepts). The statistical significance level was set at 5% ($P < 0.05$). Data are reported as means ± SD in the text. Time-mediated differences in serum lipid variables in Table 2 were calculated using ANOVA as described earlier ($P < 0.05$). Similarly, the strain differences (Table 2; normolipemic versus hypercholesterolemic) for each lipid variables were analyzed as one block using ANOVA and by Fisher's LSD, and differences were considered significant at $P < 0.001$.

RESULTS

The gain in body weight in the HH swine did not differ between the treatment groups and their corresponding control group, although feed efficiency was greater in swine fed *d*-P_{21}-T3 and *d*-P_{25}-T3 (**Table 1**). The gain in body weight in the normolipemic swine did not differ from the HH swine groups (Table 1).

Serum total-chol concentrations were significantly increased 10, 55 and 63% ($P < 0.05$) after feeding control diet to HH swine for 21, 42 and 70 d, respectively, compared with zero time (**Table 2**). In normolipemic swine, these values were 10, 19 and 24% ($P < 0.05$) at 21, 42 and 70 d, respectively, compared with zero time (Table 2). The serum LDL-chol levels for HH swine and normolipemic swine were 76 and 25% ($P < 0.05$), respectively, at 70 d compared with zero time (Table 2).

Feeding of TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3 to HH swine for 42 d significantly decreased serum total-chol concentrations by 32, 34, 36 and 38% ($P < 0.01$), respectively, compared with the concentration in the HH swine control group (**Table 3**). The levels of LDL-chol were decreased by 35, 38, 40 and 43% ($P < 0.01$), respectively, compared with the HH swine control group, whereas HDL-chol levels did not differ among groups (Table 3).

TABLE 1

Effects of the tocotrienol-rich fraction from stabilized and heated rice bran (TRF_{25}) and its components on weight gain, feed consumption, feed conversion and feed efficiency (for 42 d) in normolipemic and hereditary hypercholesterolemic swine[1]

Animal and diets	Body weight 20 wk	Body weight 26 wk	Total weight gain	Unit feed consumed	Feed conversion ratio	Feed efficiency	Body weight 36 wk
	kg		*kg/42 d*		*kg gain/kg feed*	*%*	*kg/70 d*
Normolipemic swine							
control diet (CD)	66.3 ± 7.4	82.5 ± 8.9	16.2	16.4	0.99		116.2 ± 9.3
Hypercholesterolemic swine							
CD	64.9 ± 8.8	81.4 ± 10.1	16.5	18.3	0.90	100	115.2 ± 14.4[2]
CD + TRF_{25}[3]	65.3 ± 7.9	81.1 ± 9.8	15.8	17.0	0.93	103	116.3 ± 16.9
CD + γ-T3[3]	65.1 ± 8.6	80.7 ± 8.9	15.6	17.1	0.91	101	118.5 ± 12.2
CD + *d*-P_{21}-T3[3]	64.7 ± 9.2	80.9 ± 9.9	16.2	16.7	0.97	107	117.4 ± 15.8
CD + *d*-P_{25}-T3[3]	65.4 ± 9.4	81.2 ± 9.4	15.2	17.2	0.92	110	114.9 ± 14.2

[1] Data expressed as means ± SD, $n = 3$.
[2] Data expressed as means ± SD, $n = 2$.
[3] Control diet was supplemented with 50 μg either TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3 per g.
γ-T3, γ-tocotrienol; *d*-P_{21}-T3, desmethyl tocotrienol; *d*-P_{25}-T3, didesmethyl tocotrienol.

HH swine fed TRF_{25} had serum total- and LDL-chol concentrations that did not differ from those in HH swine fed γ-T3, *d*-P_{21}-T3 and *d*-P_{25}-T3. However, the ratios of HDL-chol to total chol and HDL-chol to LDL-chol in the treatment groups generally differed from the control group. The HDL-chol/total chol ratios were greater in swine fed TRF_{25}, γ-T3, *d*-P_{21}-T3 and *d*-P_{25}-T3 by 39, 59, 68 and 81% ($P < 0.01$), whereas the HDL-chol/LDL-chol ratios were increased by 44, 70, 80 and 95% ($P < 0.05$), respectively (Table 3). Similarly, the total chol/HDL-chol and LDL-chol/HDL-chol ratios in the four HH treatment groups were significantly lower than the HH swine control group. The ratios of total chol/HDL-chol compared with control HH swine group were lowered with TRF_{25} (30%), γ-T3 (39%), *d*-P_{21}-T3 (42%) and *d*-P_{25}-T3 (46%) ($P < 0.01$), whereas the LDL-chol/HDL-chol ratios were lowered to 31, 41, 44 and 49% ($P < 0.05$), respectively, compared with the control group (Table 3).

The reduction in total-chol and LDL-chol concentrations in HH swine fed TRF_{25}, γ-T3, *d*-P_{21}-T3 and *d*-P_{25}-T3 were more pronounced after 42 d of feeding experimental diets than after 21 d of feeding. Serum total-chol at 21 d was lower in swine fed TRF_{25}, γ-T3, *d*-P_{21}-T3 and *d*-P_{25}-T3 by 12, 10, 14 and 17% ($P < 0.05$), and LDL-chol was lower by 13, 14, 17 and 19% ($P < 0.05$), respectively, compared with the control group (data not shown).

Levels of apoA1 did not differ among groups (**Table 4**). apoB was 20–28% lower ($P < 0.01$), TG were 15–19% lower ($P < 0.05$), PF4 was 12–24% lower and TXB_2 was 11–18% lower ($P < 0.01$) in treated HH swine than in control HH swine (Table 4). The maximum effects on these variables were observed in the group fed *d*-P_{25}-T3. The four treatment groups were significantly different ($P < 0.05$) from the control group, although they were not different from one another.

Serum glucose was 22–25% lower ($P < 0.01$) in treated HH swine compared with control group (**Table 5**). Decreases of 11–17% ($P < 0.05$) were observed in the levels of glucagon. Insulin was doubled ($P < 0.01$) in treated groups relative to the controls (Table 5).

The results for the hepatic activities of HMG-CoA reductase, cholesterol 7α-hydroxylase and the levels of cholesterol

TABLE 2

Serum total, HDL and LDL cholesterol levels in normolipemic and hereditary hypercholesterolemic swine fed the control diet for 21, 42 or 70 d[1,2]

Nutritional state (control diet)	Baseline	21 d	42 d	70 d
		mmol/L		
Total cholesterol				
Normolipemic swine	3.08 ± 0.13[c] (100)[3]	3.40 ± 0.14[b] (110)	3.68 ± 0.11[a] (119)	3.82 ± 0.12[a] (124)
Hypercholesterolemic swine	5.95 ± 0.41[b] (100)	6.52 ± 0.35[b] (110)	9.23 ± 0.23[a] (155)	9.69 ± 0.36[a] (163)[4]
HDL cholesterol				
Normolipemic swine	0.44 ± 0.03[b] (100)	0.47 ± 0.05[b] (107)	0.52 ± 0.06[a,b] (118)	0.58 ± 0.04[a] (132)
Hypercholesterolemic swine	0.54 ± 0.03[b] (100)	0.62 ± 0.04[b] (115)	0.73 ± 0.15[a,b] (135)	0.79 ± 0.09[a] (146)[4]
LDL cholesterol				
Normolipemic swine	2.46 ± 0.14[b] (100)	2.86 ± 0.13[a] (116)	2.92 ± 0.21[a] (119)	3.07 ± 0.26[a] (125)
Hypercholesterolemic swine	4.98 ± 0.25[c] (100)	5.60 ± 0.28[b] (112)	8.33 ± 0.27[a] (167)	8.75 ± 0.34[a] (176)[4]

[1] Feeding periods were 21, 42 or 70 d. Blood was collected at 0800 h after overnight food deprivation (12 h) at the end of each feeding period.
[2] Data expressed as means ± SD, $n = 3$. Values in rows with a different superscript letter are significantly different at $P < 0.05$.
[3] Percentages of treatment values compared with baseline control value are given in parentheses.
[4] Data expressed as means ± SD, $n = 2$.

TABLE 3

Effects of the tocotrienol-rich fraction from stabilized and heated rice bran (TRF_{25}) and its components on serum cholesterol (chol) concentrations in 26-wk-old hereditary hypercholesterolemic swine[1,2]

Nutritional state	Total chol	HDL chol	LDL chol	Ratio			
				HDL-chol/total-chol	HDL-chol/LDL-chol	Total-chol/HDL-chol	LDL-chol/HDL-chol
	mmol/L						
Control diet (CD)	9.23 ± 0.23^{a} (100)[3]	0.73 ± 0.15^{a} (100)	8.33 ± 0.27^{a} (100)	0.079 ± 0.02^{c} (100)	0.088 ± 0.02^{b} (100)	13.08 ± 2.69^{a} (100)	11.41 ± 2.65^{a} (100)
CD + TRF_{25}[4]	6.29 ± 0.38^{b} (68)	0.69 ± 0.10^{a} (95)	5.45 ± 0.52^{b} (65)	0.110 ± 0.01^{b} (139)	0.127 ± 0.03^{a,b} (144)	9.15 ± 0.88^{b} (70)	7.89 ± 1.73^{b} (69)
CD + γ-T3[4]	6.05 ± 0.49^{b} (66)	0.77 ± 0.11^{a} (105)	5.14 ± 0.43^{b} (62)	0.126 ± 0.01^{a,b} (159)	0.150 ± 0.03^{a} (170)	7.94 ± 0.50^{b} (61)	6.68 ± 1.31^{b} (59)
CD + *d*-P_{21}-T3[4]	5.95 ± 0.44^{b} (64)	0.79 ± 0.10^{a} (108)	5.01 ± 0.51^{b} (60)	0.133 ± 0.01^{a,b} (168)	0.158 ± 0.03^{a} (180)	7.54 ± 0.53^{b} (58)	6.34 ± 1.36^{b} (56)
CD + *d*-P_{25}-T3[4]	5.74 ± 0.33^{b} (62)	0.82 ± 0.09^{a} (112)	4.78 ± 0.37^{b} (57)	0.143 ± 0.02^{a} (181)	0.172 ± 0.01^{a} (195)	7.03 ± 1.22^{b} (54)	5.83 ± 0.23^{b} (51)
ANOVA (*P*-value)	0.0001	NS	0.0001	0.0025	0.0224	0.0028	0.0133

[1] Feeding period was 42 d. Blood was collected at 0800 h after overnight food deprivation (12 h) at the end of the feeding period.
[2] Data expressed as means ± SD, $n = 3$. Values in columns with a different superscript letter are significantly different at $P < 0.05$.
[3] Percentages of treatment values compared with control value are in parentheses.
[4] Control diet was supplemented with 50 μg of either TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3 per g.
γ-T3, γ-tocotrienol; *d*-P_{21}-T3, desmethyl tocotrienol; *d*-P_{25}-T3 = didesmethyl tocotrienol; HDL, high density lipoprotein; LDL, low density lipoprotein; NS, not significant.

and total fatty acid compositions of various tissues are considered preliminary because analyses were conducted on samples obtained from a single pig fed each of the diets ($n = 1$). Normolipidemic swine ($n = 3$, an average of nine analyses) fed the control diet were included for comparison. Dietary supplementation of tocotrienols significantly reduced the induced (by food deprivation and refeeding regimen; Leveille et al. 1975) enzymatic activity of HMG-CoA reductase compared with HH swine controls (**Table 6**). However, the activity of cholesterol 7α-hydroxylase did not appear to be affected by any of these treatments compared with HH swine controls (Table 6). The hepatic activity of HMG-CoA reductase in the normolipemic swine controls was higher than the in HH swine controls (Table 6).

Preliminary data on the effect of tocotrienols in lowering cholesterol were also apparent in the various tissues tested after the experimental diet was fed for 42 d. Tissue cholesterol was lower in the liver, adipose tissue, intestine and muscles of the HH swine fed TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3 compared with HH swine controls (data not shown). The levels of cholesterol in all of these tissues of the normolipemic swine control group ($n = 3$) were lower than those of the HH swine controls (data not shown). Total fatty acids in liver and loin muscle were lower in the treated HH swine than in the HH swine controls. Myristic, palmitic, palmitoleic, palmitelaidic, oleic, linoleic and arachidonic acids were lower in the liver of the treated HH swine, whereas stearic and linolenic acids were greater than controls. In the loin muscle, only palmitic, stearic and oleic acids were found, and they were lower in treated swine than in control swine (data not shown).

Two pigs (one male and one female) in each group were transferred to the corn-soybean control diet for 10 wk after being fed the experimental diets for 6 wk. The reduced levels of serum total-chol and LDL-chol persisted even after the control diet was fed for 10 wk in all of the treatment groups compared with control group. The serum total-chol concentrations (in mmol/L) in HH swine after being fed control or treatment diets for 42 d were 9.23 for the control group, 6.29 for TRF_{25}, 6.05 for γ-T3, 5.95 for *d*-P_{21}-T3 and 5.74 for *d*-P_{25}-T3 ($n = 3$) (Table 3). The serum total-chol concentra-

TABLE 4

Effects of the tocotrienol-rich fraction from stabilized and heated rice bran (TRF_{25}) and its components on serum lipid analyses in 26-wk-old hereditary hypercholesterolemic swine[1,2]

Nutritional state	Apolipoprotein A1	Apolipoprotein B	Triglycerides	Platelet factor 4	Thromboxane B_2
	g/L				*ng/L*
Control diet (CD)	0.251 ± 0.013^{a} (100)[3]	1.48 ± 0.10^{a} (100)	0.86 ± 0.12^{a} (100)	0.25 ± 0.01^{a} (100)	0.84 ± 0.04^{a} (100)
CD + TRF_{25}[4]	0.261 ± 0.017^{a} (104)	1.18 ± 0.06^{b} (80)	0.75 ± 0.03^{b} (82)	0.22 ± 0.01^{b} (88)	0.74 ± 0.03^{b} (88)
CD + γ-T3[4]	0.262 ± 0.014^{a} (104)	1.14 ± 0.12^{b} (77)	0.70 ± 0.01^{b} (81)	0.21 ± 0.01^{b,c} (80)	0.72 ± 0.02^{b} (86)
CD + *d*-P_{21}-T3[4]	0.267 ± 0.019^{a} (106)	1.12 ± 0.09^{b} (76)	0.73 ± 0.02^{b} (85)	0.20 ± 0.02^{b,c} (76)	0.75 ± 0.03^{b,c} (89)
CD + *d*-P_{25}-T3[4]	0.267 ± 0.019^{a} (106)	1.07 ± 0.07^{b} (72)	0.70 ± 0.01^{b} (81)	0.20 ± 0.02^{b,c} (76)	0.69 ± 0.03^{c} (82)
ANOVA (*P*-value)	NS	0.0017	0.0303	0.0045	0.0009

[1] Feeding period was 42 d. Blood was collected at 0800 h after overnight food deprivation (12 h) at the end of the feeding period.
[2] Data expressed as means ± SD. $n = 3$. Values in columns with a different superscript letter are significantly different at $P < 0.05$.
[3] Percentages of treatment values compared with control value are in parentheses.
[4] Control diet was supplemented with 50 μg of either TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3 per g.
γ-T3, γ-tocotrienol; *d*-P_{21}-T3, desmethyl tocotrienol; *d*-P_{25}-T3, didesmethyl tocotrienol; NS, not significant.

TABLE 5

Effects of the tocotrienol-rich fraction from stabilized and heated rice bran (TRF_{25}) and its components on the levels of serum glucose and insulin and plasma glucagon in 26-wk-old hereditary hypercholesterolemic swine[1,2]

Nutritional state	Glucose	Insulin	Glucagon
	mmol/L	*pmol/L*	*ng/L*
Control diet (CD)	5.29 ± 0.30[a] (100)[3]	75.37 ± 5.35[b] (100)	330.41 ± 17.68[a] (100)
CD + TRF_{25}[4]	4.08 ± 0.44[b] (77)	165.75 ± 10.12[a] (220)	295.36 ± 14.02[b] (89)
CD + γ-T3[4]	4.10 ± 0.39[b] (78)	152.18 ± 9.68[a] (202)	285.41 ± 18.12[b] (86)
CD + *d*-P_{21}-T3[4]	4.07 ± 0.39[b] (77)	153.91 ± 19.32[a] (204)	277.76 ± 20.36[b] (84)
CD + *d*-P_{25}-T3[4]	3.97 ± 0.39[b] (75)	149.90 ± 18.88[a] (199)	274.41 ± 9.09[b] (83)
ANOVA (*P*-value)	0.0090	0.0001	0.0116

[1] Feeding period was 42 d. Blood was collected at 0800 h after overnight food deprivation (12 h) at the end of the feeding period.
[2] Data expressed as means ± SD, $n = 3$ swine. Values in columns with a different superscript letter are significantly different at $P < 0.05$.
[3] Percentages of treatment values compared with control value are in parentheses.
[4] Control diet was supplemented with 50 μg of either TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3 per g.
γ-T3, γ-tocotrienol; *d*-P_{21}-T3, desmethyl tocotrienol; *d*-P_{25}-T3, didesmethyl tocotrienol.

tion (in mmol/L) after transferring all of the groups to the corn-soybean control diet for 10 wk (70 d) were 9.69 for the control group, 7.19 for TRF_{25}, 7.39 for γ-T3, 7.14 for *d*-P_{21}-T3 and 6.95 for *d*-P_{25}-T3 ($n = 2$). The reductions were 74, 76, 74 and 72% in the treatment groups compared with the control group. These reductions were slightly lower than those reported in Table 3. Similarly reduced serum LDL-chol levels persisted in the HH swine treatment groups (data not shown). These results suggest that the tocotrienols may have remained in the bloodstream over extended periods of time as reported earlier (Qureshi et al. 1991a, 1991b and 1997).

TABLE 6

Effects of the tocotrienol-rich fraction from stabilized and heated rice bran (TRF_{25}) and its components on the enzymatic activities of cholesterol metabolism in 26-wk-old normolipemic and hereditary hypercholesterolemic swine[1]

Nutritional state	HMG-CoA reductase[2]	Cholesterol 7α-hydroxylase[3]
	pmol/(min · mg) microsomal protein	*nmol/(min · mg) microsomal protein*
Normolipemic swine[4]		
Control diet	567.2 ± 27.3	6.5 ± 0.16
Hypercholesterolemic swine[5]		
Control diet (CD)	391.4 (100)[6]	5.07 (100)
CD + TRF_{25}[7]	285.1 (74)	5.02 (99)
CD + γ-T3[7]	280.9 (73)	4.98 (98)
CD + *d*-P_{21}-T3[7]	275.8 (70)	4.93 (97)
CD + *d*-P_{25}-T3[7]	269.4 (69)	4.98 (98)

[1] Feeding period was 42 d. At the end of the feeding period, swine were deprived of food for 48 h and refed for 48 h, and one male pig (randomly selected) from each group was killed at 0800 h.
[2] In pmol of mevalonate/min/mg microsomal proteins.
[3] In nmol of [^{14}C]cholesterol converted into [^{14}C]7α-hydroxycholesterol/min/mg microsomal protein.
[4] Data expressed as means ± SD, $n = 3$.
[5] Data expressed as means ± SD, $n = 1$.
[6] Percentages of treatment values compared with control value are in parentheses.
[7] Control diet was supplemented with 50 μg of either TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3 per g.
γ-T3, γ-tocotrienol; *d*-P_{21}-T3, desmethyl tocotrienol; *d*-P_{25}-T3, didesmethyl tocotrienol.

DISCUSSION

This study confirms that novel tocotrienols induce favorable changes in lipid and lipoprotein profiles in a strain of swine exhibiting HH, which was reported earlier in humans (Qureshi et al. 1997). Hypercholesterolemia in these swine results from a hereditary defect in catabolism that leads to an elevation of LDL. The increase in cholesterol-rich LDL particles causes atherosclerotic arteries similar to those observed in humans (Checovich et al. 1988). The life-span of HH swine is only one third that of normolipemic swine (Prescott et al. 1995).

Although body weight gains were similar in HH and normolipemic swine fed corn-soybean control diet from 20 to 36 wk (65–115 kg in all the groups), serum total-chol (or LDL-chol) concentrations increased far more in HH swine (63%) compared with normolipemic swine (24%) during the 16-wk growth period (Table 2). The plasma lipids and lipoproteins in HH swine differed significantly from those of normolipemic swine (Qureshi et al. 1991a). Tocotrienols are more effective in hypercholesterolemic individuals, which explains the large magnitude of their effects in the HH swine (Qureshi et al. 1991a, 1991b, 1997 and 1999).

The significant decline in the concentrations of plasma TXB_2 and PF4 in swine fed TRF_{25} and its novel tocotrienols (*d*-P_{21}-T3 and *d*-P_{25}-T3) is of great interest, because these results show that TRF_{25} and its components not only produce favorable lipid profiles but also can have a protective effect on the endothelium and/or platelets. Thromboxanes induce platelet aggregation and vasoconstriction, whereas prostacyclins inhibit platelet aggregation and produce vasodilation. These results may be mediated in part through reduced prostaglandin synthesis from fatty acids. The preliminary results of the effects of TRF_{25}, γ-T3, *d*-P_{21}-T3 and *d*-P_{25}-T3 isolated from rice bran on the fatty acid compositions of various tissues of swine showed a significant decrease in levels of arachidonic acid in liver and loin muscles, which might explain the dramatic reduction in the serum TXB_2 and PF4 levels (Qureshi et al. 1999). The lowering of arachidonic acid by these novel tocotrienols in various tissues of HH swine may result in the reduction of oxygen metabolites in blood. Thus, there is an overall reduction in prostaglandins and leukotrienes, both of which are synthesized from arachidonic acid (Qureshi et al. 1999). Some investigators attribute these tocotrienol-mediated actions to their unique *in situ* antioxidant activity, which is at least threefold to fivefold better than that of α-tocopherol

(Serbinova et al. 1991). The decrease in concentrations of arachidonic acid by these novel tocotrienols may be due to the inhibition of phospholipase A_2 activity.

The changes in levels of insulin and glucagon may have mediated the decrease in glucose levels observed in this study. We found a greater insulin-to-glucagon ratio (>100%) in all the treatment groups (0.533–0.563) compared with controls (0.245). Such an effect may decrease glucose intolerance in patients with diabetes mellitus and restore the acute glucose-induced insulin response in non–insulin-dependent diabetes mellitus patients. The 100% increase in the serum insulin levels with novel tocotrienols in HH swine is probably due to their antioxidant property. The positive effects of vitamin E (tocopherol) due to its antioxidant activity on the complications of diabetes have been reported (Karpen et al. 1982 and 1985). The novel tocotrienols isolated from rice bran are 8- to 33-fold better antioxidants than tocopherols (Qureshi et al. 2000).

Hepatic HMG-CoA reductase activity was 26–31% in HH swine fed TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3. The activity of cholesterol 7α-hydroxylase was unaffected. The reductions in hepatic HMG-CoA reductase are consistent with the results reported for chickens fed TRF_{25}, γ-T3, *d*-P_{21}-T3 or *d*-P_{25}-T3 (16–36%), in which eight chickens per group were studied (Qureshi et al. 2000). Moreover, the hepatic HMG-CoA reductase of adipose tissue in the normolipemic swine ($n = 3$) control group was 27% higher, as expected, compared with the HH swine control group (Qureshi et al. 1991a). The activities of HMG-CoA reductase and cholesterol 7α-hydroxylase enzymes are generally considered to be rate-limiting steps for cholesterol and bile acids synthesis in liver, respectively. The activities of HMG-CoA reductase and cholesterol 7α-hydroxylase are regulated coordinately, but in opposite directions, by phosphorylation and dephosphorylation (Carlson et al. 1978).

Novel tocotrienols are potent cholesterol inhibitors, acting most likely through feedback inhibition. The suppression of HMG-CoA reductase requires two regulators: cholesterol and a nonsterol. The cholesterol regulator is expressed predominantly through changes in the rate of transcription of the HMG-CoA reductase gene, and the nonsterol product regulator modulates the efficiency of the translation of HMG-CoA reductase mRNA or by degradation of the enzyme itself (Brown and Goldstein 1986, Goldstein et al. 1985). As reported earlier, both the mass and activity of HMG-CoA reductase are decreased in the liver after the administration of tocotrienols, and inhibit cholesterol biosynthesis by suppressing HMG-CoA reductase through a novel post-transcriptional mechanism involving protein degradation (Parker et al. 1993).

The efficacies of the individual components γ-T3, *d*-P_{21}-T3 and *d*-P_{25}-T3 compared with TRF_{25} in these experiments are very similar, which may be attributed to either the 50 μg/g concentration being at the saturation level or the fact that swine consume large amounts of feed daily compared with other experimental models (rats and chickens), resulting in a high accumulation of tocotrienols in the body, of which only small amounts might be converted to tocopherols in this model. These findings are in sharp contrast to those for chickens (Qureshi et al. 1999). Recently, Khor et al. (1995) reported a dose-dependent inhibition (17–50%) of HMG-CoA reductase activity resulted from the administration of γ-tocotrienol for 6 d to guinea pigs. The authors reported that low doses of pure γ-tocotrienol are much more effective than the higher doses and confirmed our earlier observation that α-tocopherol increases HMG-CoA reductase activity (Khor et al. 1995, Khor and Ng 1999, Qureshi et al. 1996, Qureshi and Qureshi 1993).

The lower inhibitory effect on HMG-CoA reductase activity by tocotrienols at higher doses is due to its conversion to tocopherols in the tissue, which may counteract the effect of tocotrienols. In our earlier studies, we reported high levels of α-tocopherol in serum after feeding of TRF from palm oil or γ-tocotrienol in humans (Qureshi et al. 1991b). To confirm this result, we fed radioactive synthetic [^{3}H]γ-tocotrienol to chickens for 4 wk (2×10^6 dpm). The serum, analyzed by HPLC (Qureshi et al. 1997), showed radioactivity in α-tocopherol (3265 dpm), α-tocotrienol (5815 dpm), γ-tocopherol (4650 dpm) and γ-tocotrienol (12,926 dpm) but none in δ-tocotrienol, indicating a similar metabolic biosynthetic pathway for tocotrienols in animals and plants (Qureshi and Qureshi 1993). The enzymes of the gut bacteria in the chickens may be responsible for these conversions (Hood 1995). The conversion of [^{3}H]γ-tocotrienol into various tocols was only 1.3%; α-tocopherol accounted for ~10% of the total counts recovered after 1 mo of feeding. Although this conversion into α-tocopherol is small, it could exert a negative effect on the reduction in lipid concentrations produced by tocotrienols.

However, the results of the present study demonstrate that the reductions in serum total-chol and LDL-chol levels by tocotrienols were time dependent and that the effects were more pronounced after 42 d compared with after 21 d of feeding. Moreover, the reductions in total-chol and LDL-chol observed after 42 d persisted over 10 wk after the termination of tocotrienol feeding. This result suggests that the tocotrienols may have remained in the bloodstream and that the conversion of tocotrienols to tocopherols in this HH swine model is not as rapid as that in humans (Qureshi et al. 1997). In humans, higher levels of tocotrienols were found in the HDL moiety compared with the LDL moiety after feeding of tocotrienols for 4 wk, as reported earlier (Qureshi et al. 1997), and these tocotrienols are transported nonspecifically like other lipid-soluble compounds to different tissues by lipoproteins (Qureshi et al. 1997).

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